ORGANIZATION OF THE 5S RIBOSOMAL RNA GENES AND ADJACENT SPACER REGIONS IN THE CEREAL RUSTS AND BUNTS PATHOGENIC ON WHEAT

by
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A Thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

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LIST OF ABBREVIATIONS

DNA	Deoxyribonucleic Acid
RNA	Ribonucleic Acid
dNTP	Deoxynucleotide Triphosphate
ddNTP	Dideoxynucleotide Triphosphate
rDNA	Ribosomal DNA
rRNA	Ribosomal RNA
WSR	Wheat Stem Rust
WLR	Wheat Leaf Rust
OCR	Oat Crown Rust
OSR	Oat Stem Rust
RSR	Rye Stem Rust
IGR	Intergenic Region
IEF	Isoelectric Focusing
SDS	Sodium Dodecyl Sulfate
EDTA	Disodium Ethylene Diamine Tetraacetate
CTAB	Hexadecyltrimethylammoniumbromide
TBE	Tris-borate EDTA running buffer
TAE	Tris-acetate EDTA running buffer
DTT	Dithiothreotole
NTB	Nick Translation Buffer
DNase	Deoxyribonuclease
RNase	Ribonuclease
SSC	Sodium Chloride/Sodium Citrate Buffer
PCR	Polymerase Chain Reaction

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ABSTRACT

Total genomic DNA was isolated from three cereal stem rusts, Puccinia graminis f.sp. tritici, f.sp. secalis, f.sp. avenae, two cereal leaf rusts, P. recondita f.sp. tritici, P. coronata f.sp.avenae, and two bunts, Tilletia caries and T. controversa. This DNA was analyzed for the presence of heterogeneity in the intergenic region of the ribosomal DNA repeat unit. A 1 kb region of the repeat unit between the 26S and the 5S rRNA genes (IGR-1) was amplified using the polymerase chain reaction (PCR) and was found to be heterogeneous within each isolate of several of the rusts and variable in size between races, species and genera. In an isolate of wheat stem rust race C36(48), heterogeneity appeared to be a result of varying numbers of a 0.1 kb subrepeat (or subrepeats) in IGR-1. Nine wheat stem rust races produced a unique pattern of heterogeneity and comparisons of different isolates of the same race produced identical patterns of heterogeneity suggesting a possible rapid method for race identification in wheat stem rust. Heterogeneity and polymorphism in rye stem rust, oat stem rust, wheat leaf rust, oat crown rust, and the bunts was less pronounced than in wheat stem rust. During the course of this work the 5S rRNA gene was located and its position and orientation within the ribosomal repeat unit was established in both the rusts and bunts. As well the

nucleotide sequence of the 5S rRNA gene was determined in the bunts.

INTRODUCTION

Current methods of identifying the cereal pathogens of the species *Puccinia* (the fungus responsible for causing cereal rust) and *Tilletia* (the causal agent of the bunts) are dependant on phenotypic differences. The species *Puccinia graminis* includes several formae speciales which are essentially distinguished by their cereal hosts. Each forma specialis is separated into races as determined by its virulence/avirulence host interactions. The genus *Tilletia*, pathogenic on wheat, is separated into several species including *T. caries* and *T. controversa*. *T. caries* is responsible for the more widely distributed common bunt while *T. controversa* causes dwarf bunt which is not readily preventable and is thus a quarantine organism for many countries. At present these species are differentiated on the basis of the morphological and physiological characteristics of the teliospore as well as by virulence/avirulence formulae.

An alternative method of identifying the *Tilletia* and *Puccinia* species would be desirable. For these pathogens, the virulence/avirulence method of determination is time consuming and the question of the statistical validity of the number of host cultivars used is doubtful.

Two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) has been used to separate proteins from complex biological preparations. This process allows the detection of minute variations in charge and molecular weight of a protein. These protein variations have been used in the taxonomy of fungi (Macko et al. 1967) and other organisms. This technique has allowed the differentiation of the races of P. graminis f.sp. tritici (Howes et al. 1982 and Kim et al.1982) and to a certain extent also those of T. caries and T. controversa (Kawchuk et al. 1988). It was impossible however to differentiate races of P. graminis on the basis of 2-D PAGE and while there were some polypeptides found only in some races and collections of T. controversa and T. caries, no correlation was found between polypeptides and virulence. Thus differences can not be usefully detected at the level of proteins, the gene products. It seems the next area to examine then is at the gene level.

Over the last few years, ribosomal DNA (rDNA) repeat units have been used as a source of information for the identification of many fungal species (Klassen et al. 1987; Kohn et al. 1988; Rogers et al. 1989). The rDNA repeat unit is made up of highly conserved regions as well as variable regions. The conserved regions of the repeat unit include the 18S, 5.8S, and 26S ribosomal RNA (rRNA) genes. The largely nontranscribed intergenic regions, particularly the

region seperating the 18S and 26S rRNA genes, are more variable (Long and Dawid 1980). The intergenic region (IGR) of the rDNA repeat unit of Puccinia and Tilletia seperates the 3' end of the 26S rRNA gene and the 5' end of the 18S rRNA gene.

The organization of 5S rRNA genes in fungi is known to follow one of two patterns: either most or all of the 5S genes are linked with the large rRNA genes; or the 5S RNA genes are dispersed within the genome (Amici and Rollo 1991 and Long and Dawid 1980). The pattern of the 5S gene being linked to the large subunit genes has been demonstrated in at least one example of the subdivision Basidiomycotina, Pyrenophora graminea (Amici and Rollo 1991). The main thrust of this project is to determine the position and orientation of the 5S rRNA gene in several species of Tilletia and Puccinia, to sequence the 5S rRNA gene and flanking regions in the two Tilletia species and to compare the flanking regions sequence with that of several species of Puccinia.

LITERATURE REVIEW

The organism Tilletia caries is a member of the fungal subdivision Basidiomycotina. T. caries infects both spring and summer wheats, barley and certain grass species causing common bunt (also known as stinking smut or covered smut). Plants infected with common bunt may be moderately stunted but are not readily distinguished as being infected until the heads emerge. The infected heads are slender and are distinguished by their bluish green color or the maintenance of a green color for a greater time period than healthy plants. The glumes of some or of all the spikelets become conspicuously spread apart exposing the bunt balls which have replaced the kernels. They are bluish green, becoming greyish brown at maturity. The bunt balls are similar to the normal seed in shape but are usually more spherical. Upon rupturing, black powdery teliospores having a strong fishy odour are released. These teliospores remain on the seeds or in the dry soil until the cool moist soil conditions required for germination occur. Common bunt is currently controlled using resistant cultivars, clean seed, and chemical seed treatment. Most resistant cultivars are short-lived, however, due to the development of new virulent races of the fungus.

Tilletia controversa, a Basidiomycete, is the cause of dwarf bunt which infects wheat, rye, winter-sown barley as well as a variety of wild and cultivated grasses. Natural infection by dwarf bunt is common only in wheat and rye and is apparently limited to areas where winter wheat is subjected to prolonged snow cover. The symptoms of dwarf bunt resemble those of common bunt except that the infected plants achieve only one quarter to one half the size of infected plants. The infected plants also have an increased number of tillers, broader spikes and glumes which open wider.

The primary method by which plants are infected with dwarf bunt are soil-borne teliospores. The teliospores of T. controversa persist in the soil for up to ten years, have a lower optimal temperature for germination (3-8°C) than those of T. caries as well as a longer incubation period (3-10 weeks) which explains why T. controversa seems to favor regions subjected to prolonged snow cover. It has also been found that plants in the 2-3 leaf stage are most susceptible to infection, making fungicides that protect seed ineffective.

Methods that are used to control T. controversa include: the use of resistant cultivars; and the application of certain fungicides (such as polychlorobenzenes) to the soil surface after seeding. These general methods of prevention are generally ineffective which explains why T. controversa is a quarantine organism for many countries. This means that certification that shipments are free of this pathogen must be presented.

For this certification to be effective the dwarf bunt and the common bunt fungus must be readily distinguishable. Currently this is achieved by observation of characteristics of the teliospore. The teliospores of T. controversa are similar to those of T. caries with the exception that the teliospores of T. controversa are less likely to collapse. They both have reticulate walls but with T. controversa the reticulations are typically broader, deeper and shrouded by a gelatinous sheath. Using fluorescent microscopy, the teliospores of T. controversa tend to fluoresce and appear lighter. In addition the bunt balls of T. controversa tend to be smaller and rounder. Generally these methods of identification are either unreliable or too time consuming.

Another cereal pathogen responsible for great economic losses today and throughout history is rust. The rusts are named for the dry, dusty, yellow-red or black spots and stripes (also known as sori, pustules or uredia) that erupt through the epidermis of infected plants. Different species of the rust fungus may infect any aerial portion of the plant, such as the crown (eg Puccinia coronata is responsible for oat crown rust), the leaf (eg Puccinia recondita is responsible for wheat leaf rust) or the stem (eg Puccinia graminis f.sp. tritici causes wheat stem rust). The symptons of the rusts are most obvious in spring and summer but may occur at any time after the seedlings emerge.

The rusts characteristically have a complex life cycle including alternate hosts and as many as five spore stages (three spore forms on the small grains or grasses and two on the alternate host where it exists). The most important of the spores are the urediospores which appear in the late spring and early summer. The urediospores emerge from the uredia which have broken through the epidermis of the plant and are the only spores in the life cycle of the fungus capable of reinfecting the cereal host. It is the urediospores which enable repeated cycles of the disease to spread from field to field and to survive from year to year. The different species of Puccinia are separated into races which are identified by patterns of pathogenicity on

differential hosts. New races are continually surfacing because of the pathogen's ability to mutate and sexually reproduce. The methods used to control the rusts are fungicides, the use of resistant cultivars, destruction of the alternate hosts, and the growth of different cultivars within a production area to prevent cross infection.

Currently the method of choice of intraspecifically differentiating between the rust races and the bunt races (even between T. controversa and T. caries) is to infect a series of cultivars of the host plant and to determine a virulence/avirulence equation.

Single genes for pathogen resistance have been identified (particularily in wheat for the rusts). These single genes have been transferred into host plants creating single-gene lines which are used as differential hosts for studies of virulence. Host/pathogen systems involve gene-for-gene interactions in that a specific gene for resistance in the host is matched by a corresponding gene for avirulence in the parasite.

The virulence/avirulence pattern of a culture is determined by inoculating a selected group of host plants of differing single-gene lines for resistance to the pathogen (Roelfs 1984). The pathogen will infect each plant

differently because of each plant's ability to resist infection as determined by its individual genotype for resistance. If the plant is more resistant to one race of pathogen there will be little or no infection seen and if the plant is less resistant a greater extent of infection will be the result. A race is determined when a group of biotypes have a similar virulence/avirulence pattern on a selected group of host plants. Unfortunately this is a time consuming process and negative results, reflected by little or no infection of the plant, do not always indicate resistance.

Over the years several other methods have been attempted to improve race determination. Graham (1960) proposed a series of reagents and dyes. Four distinct outer layers in teliospores were found, each layer having a different chemical composition. The compound responsible for the foetid odor of the spores of the Tilletia species, trimethylamine, was examined (Hanna et al. 1932). The enzyme activities of esterase, sulfatase and glycosidase were explored (Yu and Trione 1983) and T. controversa was found to have a slightly greater enzyme activity compared to the other species. Polyclonal and monoclonal antibodies derived from intact spores or from various extracts obtained from intact spores were also tried, but the antibodies reacted with spores of all of the species. Isozyme studies have been

done in Puccinia identification (Burdon and Roelfs 1985). An apparent complete association of isozyme and virulence phenotype was found in an asexual population but no such association was found in the sexual population. The possibilities of two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) were also examined.

O'Farrell (1975) initially described the separation of proteins from complex biological mixtures using two independent parameters thus obtaining a uniform distribution of proteins over the entire gel. The 2D-PAGE usually involves initially separating the proteins on the basis of their charge using a low concentration polyacrylamide isoelectric focusing (IEF) gel. The second dimension involves separating the proteins by molecular weight on a sodium dodecyl sulphate (SDS) gel. 2D-PAGE is a powerful analytical technique. It has a high resolution and sensitivity and makes possible the detection of extremely small variations in protein charge and molecular weight. This method was used to try to differentiate several phytopathogenic fungi such as: P. graminis f.sp. tritici (Howes et al. 1982); P. graminis f.sp. tritici and f.sp. avenae (Kim et al. 1982); Ustilago tritici and U. nuda (Kim et al. 1984); and T. laevis, T. caries, and T. controversa (Kawchuk et al. 1988). The general conclusion was that differences were present between the races and species

examined but no differences were found that were race or species specific.

The general conclusion for all of these biochemical analyses was that even though slight intraspecific or interspecific differences may have been present, not enough information could be generated to differentiate between races or even species of these phytopathogenic fungi at the level of the gene product. The next step was to begin examination of the DNA itself.

Ribosomal DNA has been used as a source of information for the identification of many fungal species (Klassen et al. 1987; Kohn et al. 1988; Rogers et al. 1989). The genomic organization of the three largest rRNAs, the 26S, 18S, and 5.8S subunits, is relatively uniform among eukaryotes. These three rRNA molecules are encoded on a single transcription unit and the pre-rRNA is synthesized by RNA polymerase I and then processed into the mature rRNA. The location and orientation of the gene encoding the 5S RNA, the fourth RNA component, is variable. The 5S rRNA gene may be part of the main transcription unit or it may be located throughout the genome; this is seen in the higher eukaryotes. It has been reported that it is possible for the 5S rRNA gene to be in the opposite orientation, relative to the other genes in the ribosomal repeat, in members of the same genus (Pukkila and

Cassidy 1987)

The spacer region separating the genes and repeats of the rDNA region have been found to be variable, particularly in the area separating the 26S and 18S rRNA genes. The 5S rRNA gene has been found in this region in certain organisms, including several basidiomycetes (Duchesne and Anderson 1990).

The relatedness between biological species of Armillaria, another basidiomycete, was examined (Anderson et al. 1989) by studying the rDNA. Using restriction mapping, most of the polymorphisms seen between species were located in the intergenic region and it was found that thirteen species of Armillaria could be placed in six classes with respect to rDNA maps.

Studying DNA using restriction enzymes of course gives information only on the regions which are cut by the enzymes and on the sizes of the pieces formed by the restrictions. To get a better idea of the overall picture, DNA sequencing has come to the forefront of this type of study including phylogeny and taxonomy.

Walker and Doolittle (1982) defined two distinct clusters of Basidiomycetes on the basis of the 5S rRNA sequence. The clusters were found to correspond with the presence or absence of cell wall septal dolipores. The authors found a correlation between the sequence data and the morphological traits, and described their findings as a striking example of the utility of molecular sequence data in distinguishing reliable morphological or cytological markers for a given phylogenetic lineage. In 1983 the authors sequenced 8 more fungi from the basidiomycetes and fungi imperfecti. They found this new sequencing data to conform to the association previously found, and considering all known basidiomycete 5S rRNA sequences (at the time) five distinct clusters, each cluster consisting of one sequence are evident. Each cluster was found to differ by 20 nucleotides or more.

Blanz and Gottschalk (1984) compared the 5S rRNA sequence of a group of seven smuts with the findings of Walker and Doolittle (1982, 1983). The 5S rRNA sequence of the seven smuts studied were found to be strikingly different indicating that the smuts are not one coherent group. Several of the smuts studied did fit into the five clusters described by Walker and Doolittle, however Blanz and Gottschalk found it necessary to propose two new clusters to fit those that did not.

In general, the differentiation between fungi is difficult because of their simple morphologies and convergent characteristics. Over the last several years the classification and phylogeny of the basidiomycetes has improved with the aid of the 5S rRNA sequence (Hendricks et al. 1991; Bowman et al. 1992). The initial indications are that these sequences are valuable in defining species, however differentiating between races of a species on this basis remains to be demonstrated.

METHODS AND MATERIALS

Cultures

Pure cultures of common bunt of wheat, T. caries T23 and T7926, and of dwarf bunt of wheat T. controversa D592 and D7848 teliospores were increased on plants of one host cultivar grown under controlled conditions. Spores of T. caries were germinated on 1.5% water agar at 15°C without light. Spores of T. controversa were germinated on 2% soil extract agar at 4-7°C under approximately 2150 1X of continuous cool white fluorescent light for approximately 4 weeks (Kawchuk et al. 1988). The germination products were transferred into 30 mL of Zscheile's MT2 medium. The suspension culture which resulted after incubation for 7-10 days in the MT2 medium were harvested by centrifugation at 7000 rpm (Sorvall SS-34 rotor) for 10 min at 4°C, washed with double-distilled water and lyophilized (Kim et al. 1987).

Pure cultures of wheat stem rust (WSR) P. graminis f.sp. tritici races C1(17), C10(15B-1), C17(56), C20(38), C36(48), C45(56A), C50(15B-5), C53(15B-1L), and C57(32) were each derived from a single pustule. The cultures were increased by injecting urediospores into adult wheat plants (Little Club) grown in isolation in a greenhouse (Kim and

Howes 1987). Upon harvesting, the spores were stored in liquid nitrogen. Other cereal rusts used were: wheat leaf rust (WLR) P. recondita f.sp. tritici, races Lr1 and Lr9; oat crown rust (OCR) P. coronata f.sp. avenae races OCR 152 and OCR 169; oat stem rust (OSR) P. graminis f.sp. avenae, races NA8 and NA28; and rye stem rust (RSR) P. graminis f.sp. secalis races R4 and R10. Urediospores for these other cereal rusts were produced in the same way as the WSR cultures. The urediospores were germinated as described by Kim and Howes (1987). Approximately 1 g of urediospores were evenly distributed over 4 L of water containing gramicidin (6 mg/L) in a stainless steal sink. Also in the sink were 2 glass dishes, each containing 500 mL nonanal solution (0.071 g/L). The urediospores were allowed to germinate for approximately 6 hrs. The urediosporelings were collected and lyophilized.

Preparation of DNA

The extraction of the DNA was as described by Kim et al. (1990). The lyophilized urediosporelings of Puccinia and germination products of the Tilletia teliospores (250 mg) were ground with dry ice in a mortar that had been precooled to 4°C. Samples were gently stirred for 15 min on ice in 20 mL ice-cold lysis buffer (150 mM NaCl, 50 mM EDTA, 10 mM Tris-HCl, pH 7.4, 30 μg/mL proteinase K) in the mortar and

then transferred to a 50 mL centrifuge tube. SDS (20%) was added to produce a final concentration of 2% and this mixture was incubated for 30 min at 65°C with gentle occasional stirring. This suspension was centrifuged at top speed in a table-top clinical centrifuge for 5 min to remove the spore walls and broken germ tube walls. The supernatant was removed and volume measured. NaCl was added to produce a concentration of 1.4 M and 0.1 volumes of 10% CTAB buffer (10% CTAB, 500 mM Tris-HCl, 100 mM EDTA, pH 8.0) was added. This solution was mixed thoroughly and incubated at 65°C for 10 min with occasional mixing. After cooling, the solution was extracted twice with an equal volume of chloroform-isoamyl alcohol (24:1 v/v) and the nucleic acids in the aqueous phase were precipitated with 2.5 volumes of 95% cold ethanol at -20°C. The nucleic acids were pelleted, redissolved in double-distilled water and treated with RNase as described by Kim et al. (1988). RNase (10 mg/mL) was added to the nucleic acid solution to produce a final concentration of 10 μg/mL. This mixture was incubated at 37°C for 1 hr then extracted once with an equal volume of phenol. Subsequent extractions were performed once each with phenol-chloroform-isoamyl alcohol (25:24:1 v/v) and chloroform-isoamyl alcohol (24:1 v/v). The nucleic acids in the aqueous phase were precipitated with 0.5 volumes ammonium acetate and 2.5 volumes ethanol.

Polymerase Chain Reaction (PCR)

Total DNA from each organism was dissolved in sterile double distilled water to a concentration of 0.3 μg/μL (as determined spectrophotometrically). The PCR was done in a reaction volume of 100 μL which included 10 μL of 10X Promega Taq DNA Polymerase Buffer (500 mM KCl, 100 mM Tris-HCl pH 9.0 at 25°C, 15 mM $MgCl_2$, 0.1% gelatin, 1% Triton X - 100), 8 μL of dNTP mix containing 2.5 mM of each nucleotide thus contributing 200 μM of each nucleotide to the final concentration of the reaction mixture, 1.5 μL (60 pmole) of each primer (as characterized in Table 1), 3.0 μL (0.9 μg) of template DNA solution, 0.5 μL (2.5 units) of Taq DNA polymerase (Promega) and 75.5 μL of sterile double distilled water. Mineral oil (approximately 50 μL) was placed over the reaction solution and then the reaction tube was subjected to a heating and cooling protocol in an ERICOMP Single Block System Easy Cycler Series temperature cycler.

The temperature cycling protocol was dependant on the primers being utilized for the amplification and was based on the step cycle program (Myers et al. 1989). For the QY region, the amplification began with the denaturation step at 93°C for 1 min, followed by annealing at 52°C for 1 min, and polymerization at 72°C for 2 min. For the QP region, the reaction mixture was denatured at 93°C for 30 sec, annealed

Table 1: Primers used for PCR amplification.

PRIMER	SEQUENCE	ORIGIN
Q	dACG CCT CTA AGT CAG AAT	Based on the 3' end of the 26S rRNA gene in yeast (Guttell and Fox 1988)
Y	dTCG CAG AGC GAA CGG GAT	Based on published 5S rRNA sequences (Wolters and Erdman 1988) and located at the 5' end of the 5S rRNA gene
N	dATC CCG TTC GCT CTG CGA	Is the compliment to Y
P	dGGC TCC CTC TCC GGA ATC	Located at the 5' end of the 18S rRNA gene
T1	dCTG CAG CAC TCA GGA TTC	Based on sequencing data of the 5S rRNA gene in Tilletia and located at the 3' end of the 5S rRNA gene

at 45°C for 1 min, and polymerized at 68°C for 5 min. An identical cycle was used for amplifying the NP and QT1 regions. This cycle involved denaturing at 93°C for 1 min, annealing at 45°C for 2 min, polymerizing at 72°C for 2 min, and an additional denaturing step of 94°C for 1 min before the program started. Each program (regardless of the primers being used) consisted of 25 repeats of each of the cycles described. Upon completion of the amplification, the products were immediately treated with CTAB (as described previously) with the exception that the incubation was done at 55°C rather than 65°C. DNA was precipitated with an equal volume of isopropanol at room temperature rather than with 2.5 volumes of ethanol at -20°C.

The DNA was centrifuged for 30 min at top speed in a table top centrifuge (microfuge), washed twice with cold 70% ethanol and dried under vacuum. The DNA to be run on agarose gels was dissolved in sterile double distilled water. The type of agarose gel used was dependant on the purpose of the gel run. For DNA quantification and for blotting purposes, the DNA was run on a 1.3% agarose gel in 1X TBE (2 mM EDTA, 90 mM Tris-borate). When the DNA was to be eluted from the gel, it was run on a 1% agarose gel in 1X TAE (40 mM Tris-acetate, 1 mM EDTA). The running buffer was identical to the buffer used to make the gel. On average, the gels were run for 400 V-hrs although this could vary depending on the size

of the DNA to be separated on the gel. A 1 kb ladder (BRL) was run on all gels as a standard (4% ladder, 17% stop buffer, 79% water), and all gels were stained for 30 min in a solution of running buffer and ethidium bromide at a concentration of 0.5 μg/mL (as described by Sambrook et al. 1989).

Restriction Endonuclease Reactions

The PCR amplified DNA as well as the total genomic DNA was restricted using the restriction endonucleases EcoRI, XbaI and MspI essentially as described by Sambrook et al. (1989). The reaction volume was 10 μL which consisted of 0.5 μL (5 units BRL) enzyme, 2 μL (2 μg when using PCR product, 5 μg when using genomic DNA) DNA, 1 μL buffer, and water to bring the volume to 10 μL. The reaction time was 3 to 4 hrs when the PCR product was being digested, and 16 to 20 hrs when genomic DNA was being digested. The reaction temperature was 37°C for both situations. The reaction was stopped with 2 μL stop solution and the reaction mixture was immediately run on an agarose gel.

Southern Blotting

The alkaline capillary transfer of DNA from an agarose gel to Zeta-Probe GT membrane (BIO-RAD) was performed as a

variation of the method described by Sambrook et al. (1989). A glass plate was placed over top of a baking dish containing 0.4 N NaOH and 0.6 M NaCl. Three layers of Whatman 3 mm paper were wrapped around the plate so that the ends were immersed in the NaOH/NaCl solution. The trimmed gel (wells and edges removed) was placed on top of this and over this was placed the membrane (presoaked in water), two layers of Whatman 3 mm paper and a stack of paper towels (all cut to the dimensions of the gel). Another glass plate was placed on top of the paper towels and a light weight on top of this. The glass dish and lower plate were covered with parafilm (except where the gel was located) and the DNA transfer was allowed to take place for 18-24 hours. Upon completion of transfer the membrane was neutralized with a 2X SSC (0.15 M NaCl, 0.015 M sodium citrate, pH 7.0) rinsed and baked at 80°C for 30 min to crosslink the DNA and membrane.

Hybridizations

A ribosomal DNA specific probe was produced by labelling pMF2 (Free et al. 1979) plasmid DNA (which contains the portion of the rDNA repeat unit of Neurospora crassa with the 18S, 5.8S, and 25S rDNA cistrons) with α-^{32}P-dATP by nick translation essentially as described by Sambrook et al. (1989). The reaction volume was 50 μL and

consisted of 1 μg pMF2 DNA, 28.5 μL water, 10 μL 5X NTB buffer (1 μM dCTP, 1 μM dGTP, 1 μM dTTP, 2.5 M MgCl2, 62.5 mM Tris-HCl pH 7.6, 2.5 μg/5μL Bovine serum albumen, 1 mM DTT), 2 μL DNase (Sigma 0.1 μg/mL), 0.5 μL DNA polymerase I (Promega 5 units as described by Richardson et al. 1964) and 5 μL α-^{32}P-dATP (1 mCi in 100 μL; Dupont). This was incubated at 15°C for 1.5 hrs. The reaction was stopped by precipitation with 0.5 vol ammonium acetate and 2.5 vol 95% ethanol at -70°C for 1 hr. The probe DNA was pelleted by centrifuging at top speed in a table top centrifuge for 30 min and was washed with cold 70% ethanol. The DNA was redissolved in 600 μL TE (10 mM Tris-Cl, 1 mM EDTA), placed in a boiling water bath for 10 min then immediately added to the blot in the prehybridizing solution, and the blot was incubated with constant agitation for 16-20 hrs at 55°C. The probe of the QY PCR product was produced in the same manner.

The blot was prehybridized in 35 mL prehybridizing solution (1% SDS, 1 M NaCl and 100 μg/mL autoclaved and boiled salmon sperm DNA) for 2 hr. After the hybridization the blot was washed twice with 2X SSC at room temperature for 5-7 min and thrice with 2X SSC, 1% SDS at 55°C for 20 min. The blots were exposed to Kodak X-OMAT film at -70°C for 1-7 days.

Elution of DNA from the gel

After staining gels with ethidium bromide, the DNA bands were visualized with ultraviolet light, cut out and frozen at -20°C. The gel plug was treated essentially as described by Hausner et al. (1992). It was placed between two layers of Parafilm and gently thawed by steady finger pressure. The expressed liquid was collected and to it was added 5 M NaCl to produce a final concentration of 1 M NaCl and 10% CTAB to produce a final concentration of 1% CTAB. This was incubated at 55°C for 10 min with gentle mixing, and upon cooling, two chloroform-isoamylalcohol (24:1 v/v) extractions were performed. This was followed by precipitation of the DNA by the addition of an equal volume of isopropanol (at room temperature).

Sequencing

The sequencing technique used was as described by Hausner et al. (1992). Approximately 1 μg (3.5 μL) of double-stranded, PCR amplified template DNA was mixed with 4 uL 0.6 N NaOH, 5 μL (8 pmole) of primer solution and 12 μL of tricine buffer (0.6 M tricine, 2% NP-40, 100 mM $MgCl_2$). This annealing mix was boiled for 3 min then immediately transferred to a -70°C ethanol bath. This mixture was thawed on ice and 4 units (0.5 μL) of T_7 polymerase (Pharmacia) in

4 μL dilution buffer, 1 μL of 100 mM dithiothreitol and 2 μL of α-^{32}P-dATP (1 mCi in 100 μL; Dupont) were added. Of this mixture 7.1 μL was added to each of four sequencing termination mixes (each consisting of 80 μM of each of dATP, dCTP, dGTP, dTTP and one of 6 μM ddATP, ddCTP, ddGTP, ddTTP; Pharmacia). After incubation for 7 min at 37°C the contents of each tube was diluted with 9 μL water and precipitated with 51 μL of ethanol solution (95% ethanol, 0.12 M sodium acetate). The DNA was pelleted by centrifugation at top speed in a table top centrifuge for 30 min and then washed with cold 70% ethanol. The pellet was resuspended in stop buffer (4% Xylene and 4% bromophenol blue in 200 mM EDTA pH 8.0 in deionized formamide) and loaded on the sequencing gel (6% polyacrylamide and 48% urea, as described by Sambrook et al. 1989). Two loadings were spaced approximately 1.5-2.0 hrs apart; this allowed determination of 200-250 nucleotide stretches. Gels were vacuum dried and exposed for 1-7 days to Kodak X-OMAT film at room temperature.

RESULTS AND DISCUSSION

The 5S rRNA gene of Tilletia and Puccinia

A method for the location of the 5S gene in the ribosomal repeat unit, developed for studies of *Pythium* species (Belkhiri et al. 1992), was applied to cereal rusts and bunts. A primer pair in which one primer recognizes a conserved region in the small or large rRNA genes and the other is specific to the 5S gene can be used as a preliminary way to locate and judge the orientation of the 5S gene. Primers used in this study and their recognition regions are described in Table 1. Amplification with QY and NP primers was taken to mean that the 5S rRNA gene was present in the IGR and found on the same strand as the 26S gene ("not inverted"). The opposite orientation ("inverted") would result in amplification with QN and YP. If one set of primers results in amplifications, the other should not. If there is no amplification with any of these primer pairs, the gene is likely absent from the IGR.

The location of the 5S rRNA gene in the bunts of wheat

Total genomic DNA of *T. caries* races T23 and T7926 and *T. controversa* races D592 and D7848 was used as a template for amplification by primer pairs described above and in

Table 1. Total genomic DNA of these four races was also used as template to amplify the NP region producing a 1.6 kb fragment. Amplification of DNA from all four races using the QY primer pair produced a 1.8 kb fragment for each race (Fig 1). In each case a single band was produced, indicating that no length heterogeneity exists. Attempts were made, unsuccessfully, to amplify the DNA with QN and YP primer pairs. This indicates that the 5S rRNA gene is in what was thought to be the IGR and on the same strand of DNA as the other ribosomal genes (Fig 2). With this in mind, the regions on either side of the 5S rRNA gene have been designated IGR-1 and IGR-2. The sizes of the IGR-1 and IGR-2 can be determined using the assumption that the 26S and 18S rRNA genes in Tilletia are approximately the same length as their counterparts in yeast. Based on this assumption the QY region contains about 0.3 kb of gene sequence contributed by portions of the 26S and 5S rRNA genes making the IGR-1 1.2 kb in length. Similarily, the NP region contains approximately 0.4 kb of gene sequence from the 18S and 5S rRNA genes making the IGR-2 1.1 kb in length. The trend for Basidiomycetes is that the IGR-2 is longer than the IGR-1 (Pukkila and Cassidy 1987) which is the opposite of what is seen here.

Figure 1. Agarose gel of PCR amplified products of Tilletia controversa. Ten uL of the 100 uL reaction volume of the PCR product was assayed in each lane of the 1.3% agarose gel. A) Agarose gel of NP and QY PCR products of T. controversa isolate D592: lane 1, BRL 1 kb ladder; lane 2, NP PCR product; lane 3, QY PCR product B) Agarose gel of the QP PCR product of 2 isolates each of T. caries and T. controversa: lane 1, BRL 1 kb ladder; lane 2, T. caries isolate T23; lane 3, T. caries isolate T7926; lane 4, T. controvrsa isolate D592; lane 5, T. controversa isolate D7848. lane 3, BRL 1kb ladder.

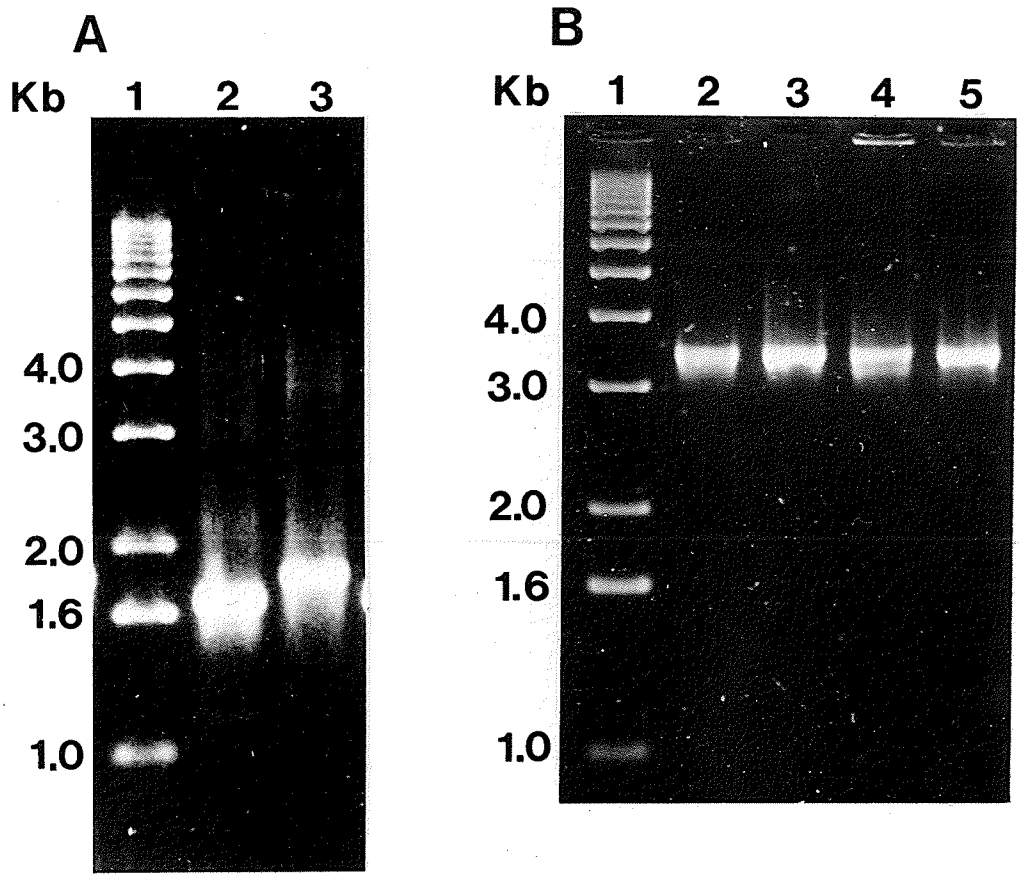
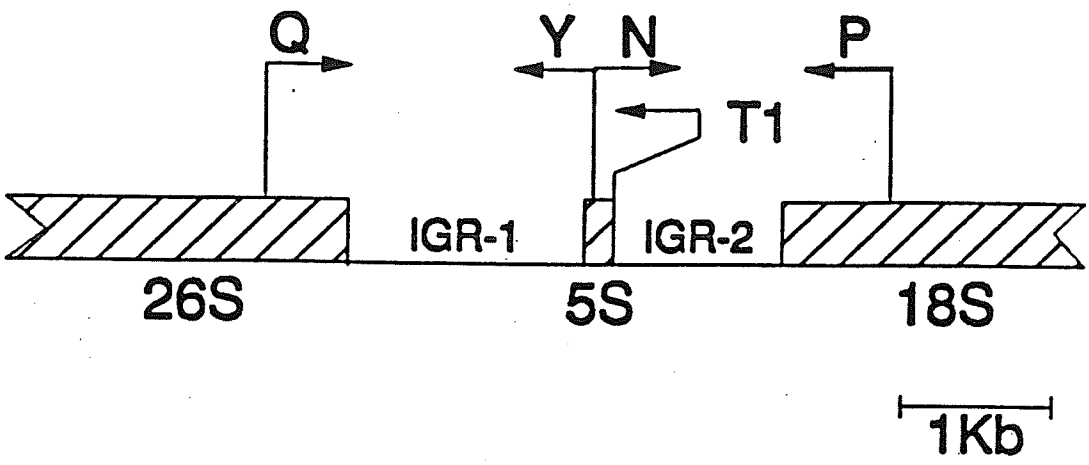

A
Kb
1
2
3
4.0
3.0
2.0
1.6
1.0
B
Kb
1
2
3
4
5
4.0
3.0
2.0
1.6
1.0

Figure 2. Location of 5S rRNA gene in T. caries and T. controversa. The shaded areas represent the 26S, 5S and 18S rRNA genes (although only portions of the 26S and 18S rRNA genes are shown). The IGR-1 is located between the 3' end of the 26S rRNA gene and the 5' end of the 5S rRNA gene. The IGR-2 is located between the 3' end of the 5S rRNA gene and the 5' end of the 18S rRNA gene. The primer annealing sites and direction of amplification (as indicated by the arrows) are shown for the primers Q, Y, N, T1 and P.


Q
Y
N
P
T1
IGR-1
IGR-2
26S
5S
18S
1Kb

The sequence of the 5S rRNA gene and flanking regions in the bunts of wheat

In order to sequence the 5S rRNA gene and flanking regions, the entire IGR was amplified using primers Q and P, Q being specific for the 26S rRNA gene and P for the 18S rRNA gene (Fig 2). The sequences of the 5S rRNA genes and flanking regions for the four races of Tilletia were determined by double-stranded sequencing of the 5S gene region using this product as template. The primer N was used to sequence the 3' end of the 5S rRNA gene and the 5' end of the IGR-2. Having obtained the sequence of the 3' end of the 5S rRNA gene, we were able to design primer T1 to be used to sequence the central region of the gene (including the N/Y region) the 5' end of the 5S rRNA gene and the 3' end of the IGR-1. The QY region was also used as template DNA for sequencing further into the IGR-1 than was possible using T1 primer, this was accomplished using Y primer which is the complement of N. These three overlapping sequences were combined to create the sequence seen in Fig 3. The sequences of the 5S rRNA genes and their flanking regions for the four Tilletia races studied were found to be identical and the 5S rRNA sequence was found to agree perfectly with the published sequence of the 5S rRNA gene in T. controversa by Walker and Doolittle (1983). Examination of the flanking regions of the 5S rRNA gene reveal TATA sequences beginning

Figure 3. DNA sequence of the 5S rRNA gene region of two races each of T. controversa (D592 and D7848) and T. caries (T23 and T7926). The segment corresponding to the published 5S rRNA gene sequence (Walker and Doolittle 1983) of T. controversa is underlined. The upstream TATA sequence is indicated by dots over the bases.

```
T23     GTGGTCAATGCGGCAGCATGCGGCGACGCGTCCTGACATGTGTCCTCTCA   50
T7926   GTGGTCAATGCGGCAGCATGCGGCGACGCGTCCTGACATGTGTCCTCTCA   50
D7848   --------------------------------------------------    0
D592    --------------------------------------------------    0

T23     TATTGTATCCATTGTCCCGCCCATTGATATGAATGAGGTGCGGGCCAGAT   100
T7926   TATTGTATCCATTGTACCGCCCATTGATATGAATGAGGTGCGGGCCAGAT   100
D7848   --------------------CCATTGATATGAATGAGGTGCGGGCCAGAT    30
D592    ------------------------------------TGCGGGCCAGAT    12

T23     CATTGTCCGACCCATTGAAATGATATGAGGTACGGACCATCCATAGCCCG   150
T7926   CATTGTCCGACCCATTGAAATGATATGAGGTACGGACCATCCATAGCCCG   150
D7848   CATTGTCCGACCCATTGAAATGATATGAGGTACGGACCATCCATAGCCCG    80
D592    CATTGTCCGACCCATTGAAATGATATGAGGTACGGACCATCCATAGCCCG    62
                              .... .... ....
T23     GCATTGTCCCAGCCATCATTATAGTATAATATATACAACAGACCGGAAAG   200
T7926   GCATTGTCCCAGCCATCATTATAGTATAATATATACAACAGACCGGAAAG   200
D7848   GCATTGTCCCAGCCATCATTATAGTATAATATATACAACAGACCGGAAAG   129
D592    GCATTGTCCCAGCCATCATTATAGTATAATATATACAACAGACCGGAAAG   112

T23     AAAATTCATCTGCGGCCATAGAACCTTGAAAGCACCGCATCCCGTCCGAT   250
T7926   AAAATTCATCTGCGGCCATAGAACCTTGAAAGCACCGCATCCCGTCCGAT   250
D7848   AAAATTCATCTGCGGCCATAGAACCTTGAAAGCACCGCATCCCGTCCGAT   179
D592    AAAATTCATCTGCGGCCATAGAACCTTGAAAGCACCGCATCCCGTCCGAT   162

T23     CTGCGAAGTTAACCAAGGTATCGCTCAGTTAGTACTGCGGTGGGGGACCA   298
T7926   CTGCGAAGTTAACCAAGGTATCGCTCAGTTAGTACTGCGGTGGGGGACCA   300
D7848   CTGCGAAGTTAACCAAGGTATCGCTCAGTTAGTACTGCGGTGGGGGACCA   229
D592    CTGCGAAGTTAACCAAGGTATCGCTCAGTTAGTACTGCGGTGGGGGACCA   212

T23     CGCGGGAATCCTGAGTGCTGCAGTTTTGCTCTTTTGCTTGATCCTTTTTG   348
T7926   CGCGGGAATCCTGAGTGCTGCAGTTTTGCTCTTTTGCTTGATCCTTTTTG   350
D7848   CGCGGGAATCCTGAGTGCTGCAGTTTTGCTCTTTTGCTTGATCCTTTTTG   279
D592    CGCGGGAATCCTGAGTGCTGCAGTTTTGCTCTTTTGCTTGATCCTTTTTG   262

T23     CTTATCCTTTATTTTTATCGCTGTTGACTATTTTTACTGCCCCGGACCAT   398
T7926   CTTATCCTTTATTTTTATCGCTGTTGACTATTTTTACTGCCCCGGACCAT   400
D7848   CTTATCCTTTATTTTTATCGCTGTTGACTATTTTTACTGCCCCGGACCAT   329
D592    CTTATCCTTTATTTTTATCGCTGTTGACTATTTTTACTGCCCCGGACCAT   312

T23     ACCTGTATGCTCCCCGGTCATGTCCCGGACCATGGCATGGGGGTGACATC   448
T7926   ACCTGTATGCTCCCCGGTCATGTCCCGGACCATGGCATGGGGGTGACATC   450
D7848   ACCTGTATGCTCCCCGGTCATGTCCCGGACCATGGCATGGGGGTGACATC   379
D592    ACCTGTATGCTCCCCGGTCATGTCCCGGACCATGGCATGGGGGTGACATC   362

T23     GTCATGGTACTGTCATAAGTGAGGTTG   476
T7926   GTCATGGTACTGTCATAAGTGAGGTTG   478
D7848   GTCATGGTACTGTCATAAGTGAGGTTG   407
D592    GTCATGGTACTGTCATAAGTGAGGTTG   390
```

38 bp upstream of the putative 5' terminus of the gene and regions rich in tracts of T downstream of the gene which are characteristic of termination sequences (Amici and Rollo 1991). Both of these features have possible regulatory significance in transcription of the gene, and their presence supports the idea that the gene we have located is the functional 5S rRNA gene in Tilletia.

The location of the 5S rRNA gene in the cereal rusts

Total genomic DNA of nine races of wheat stem rust (C1(17), C10(15B-1), C17(56), C20(38), C36(48), C45(56A), C50(15B-5), C53(15B-1L), and C57(32)), two races of rye stem rust (R4 and R10), two races of oat stem rust (NA8 and 28), two races of wheat leaf rust (Lr1 and Lr9), and two races of oat crown rust (OCR 152 and OCR 169) were amplified using Y and Q primers yielding a variety of products variable in both size and number (Fig 4 and Table 2). The amplified QY products ranged in size between 0.9 and 1.5 kb, indicating that the size of the IGR-1 is within that range. In addition, the rDNA specific pMF2 probe hybridized to the QY product (Fig 4) proving that this product contains ribosomal sequence (the pMF2 probe would recognize the 5' end of the QY PCR product which corresponds to the 3' end of the 26S rRNA gene). These results provide preliminary evidence placing the 5S rRNA gene in the IGR of the rDNA repeat unit

Figure 4. Agarose gel and Southern blot of QY PCR amplified products of a variety of races of stem, leaf and crown rust. Ten μL of the 100 μL reaction volume of the PCR product was assayed in each lane of the 1.3% (w/v) agarose gel. The gel was run for 20 h at 35 V (700 Vh) and stained with ethidium bromide (A) and Southern blotted(B). The blot was probed with pMF2. Lanes 1 and 19, BRL 1kb ladder; 2-10, wheat stem rust races C1, C10, C17, C20, C36, C45, C50, C53, C57, respectively; lanes 11 and 12, rye stem rust races R4 and R10; lanes 13 and 14, oat stem rust races NA8 and NA28; lanes 15 and 16, wheat leaf rust races Lr1 and Lr9; 17 and 18, oat crown rust races 152 and 169.

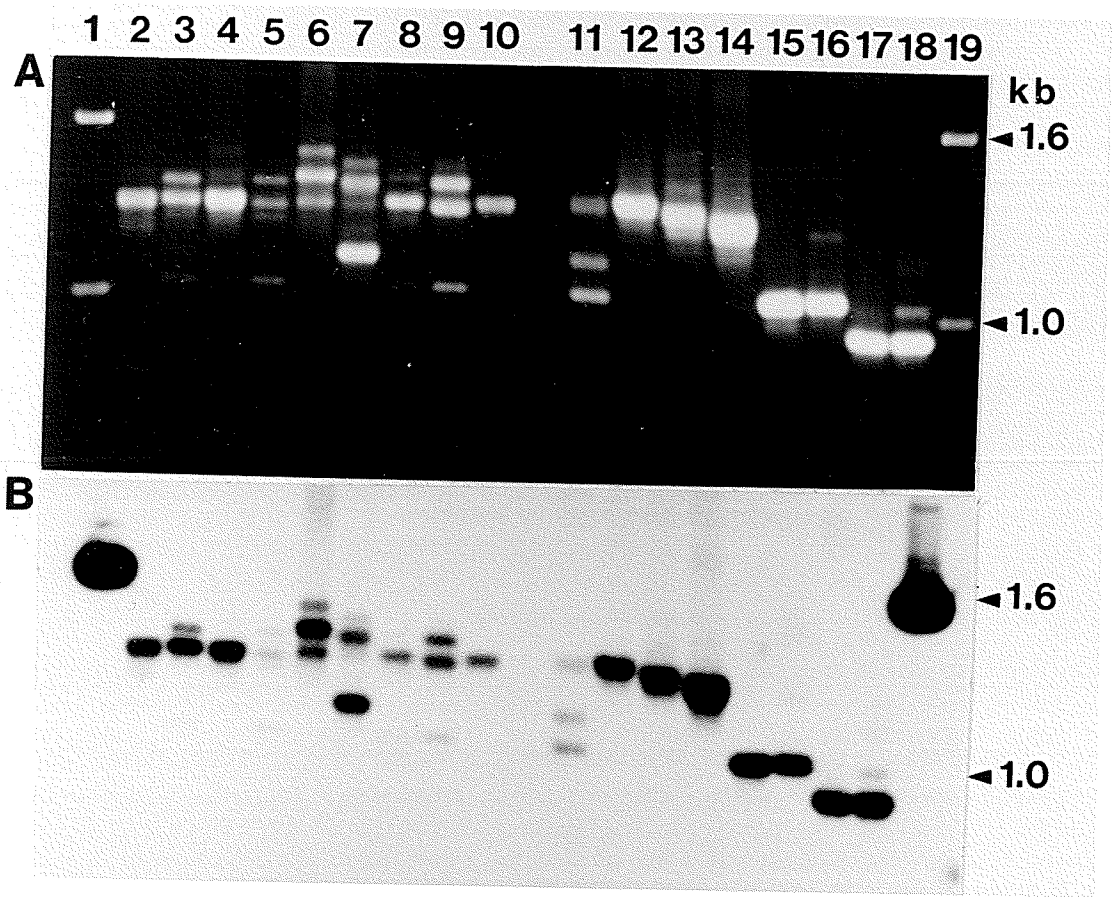

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
A
kb
1.6
1.0
B
1.6
1.0

Table 2: Sizes of PCR products in base pairs

PATHOGEN	ISOLATE	QP	QY	NP	QT1
T. caries	T23	3400	1800	1600	1900
	T7926	3400	1800	1600	1900
T. controversa	D592	3400	1800	1600	1900
	D7848	3400	1800	1600	1900
P. graminis f.sp. tritici	C1(17)		1280		
	C10(15B-1)		1280		
	C17(56)		1280		
	C20(38)		1280		
	C36(48)		1580		
			1480		
			1380		
			1280		
			1180		
			1100		
	C45(56A)		1380		
			1150		
	C50(15B-5)		1280		
	C53(15B-1L)		1380		
			1280		
	C57(32)		1280		
P. graminis f.sp. secalis	R4		1130		
			1040		
	R10		1280		
P. graminis f.sp. avenae	NA8		1280		
	NA28		1280		
P. coronata f.sp. avenae	OCR 152		900		
	OCR 169		900		
P. recondita f.sp. tritici	Lr 1		1030		
	Lr 9		1030		

and on the same strand as the other rRNA genes in all of these races and strains of P. graminis (the QY amplification in itself indicates the placement and orientation of the 5S rRNA gene as seen in Tilleyia) . This is in agreement with findings in all other Basidiomycetes except for one species of Coprinus (Pukkila and Cassidy 1987; Duchesne and Anderson 1990) which has the inverted orientation.

Heterogeneity in the IGR-1 of the cereal rusts

Heterogeneity is essentially due to the presence of varying sizes of a single region of a gene or spacer. Buchko and Klassen (1990) localized heterogeneity in Pythium ultimum in the IGR using PCR. During work to determine the location of the 5S rRNA gene, heterogeneity was observed in the QY PCR amplified product of C36(48) but not C1(17) (Fig 4). That is, a number of products, variable in size were found to make up the QY amplified product of total genomic DNA from C36(48). The QY amplification of C1(17) however produced only one product. The race C36(48) yielded a set of three major fragments (of 1.28, 1.38, and 1.48 kb in size) and three minor fragments (of 1.10, 1.18, and 1.58 kb in size), while the single PCR product of race C1(17) yielded a fragment of the size 1.28 kb (Table 2, Fig 4).

The discovery of heterogeneity in the QY product of C36(48) led to the examination of the QY products of various other species of Puccinia and races of these species. These included: the wheat stem rust races C10, C17, C20, C45, C50, C53, and C57; the rye stem rust races R4 and R10; the oat stem rust races NA8 and NA28; the wheat leaf rust races Lr1 and Lr9; and the oat crown rust races OCR 152 and OCR 169. These QY products, along with C1 and C36 were run on an agarose gel to compare banding patterns and the bands were probed with pMF2 to verify that the QY product was ribosomal in origin. The results are shown in Fig 4B (the pMF2 probe recognizes the 26S portion of the QY product) and the sizes of the PCR products are described in Table 2. Each of the nine wheat stem rust races examined was found to produce a unique pattern of heterogeneity with the QY PCR product ranging in size from 1.02 to 1.58 kb. The pattern of heterogeneity in the remaining species of the rusts and bunts was found to be identical. The two rye stem rust races were found to produce a single product although of differing sizes (R4 produced a 1.10 kb fragment while R10 produced a 1.02 kb fragment). The major bands of the two oat stem rust races were found to differ but slightly and the QY PCR product of race NA28 included a minor band not seen in NA8. Because of this variability in the QY product of wheat stem rust, there is the possibility of using PCR as a tool for differentiating races of wheat stem rust. Similarily,

different species of the rusts may also be differentiated in this manner. It is seen however, that the wheat leaf rusts and oat crown rusts can not be differentiated on the basis of the QY PCR product even though it has been previously shown that they can be distinguished on the basis of other molecular markers such as polypeptide electrophoretic behavior (Kim et al. 1985).

To try to further localize the region of heterogeneity, the QY PCR product of C36(48) was restricted with MspI, EcoRI and MspI plus EcoRI (Fig 5). The EcoRI digestion was found to be identical to the unrestricted product, however the MspI and the MspI/EcoRI double digestions were identical resulting in the reduction in size of each band of the heterogeneous QY PCR product by 0.23 kb as compared to the unrestricted sample. The QY PCR fragments were probed with pMF2 to prove their rDNA origin. All of the bands were homologous to the probe with the exception of the 0.23 kb fragments excised by MspI, which did not contain any regions of homology to the pMF2 plasmid.This could be predicted from the physical map of P. graminis f.sp tritici race C36(48) (Fig 6). The excision of the QY PCR product by MspI to reduce all three bands of the C36(48) QY region indicates that the region of heterogeneity is located upstream of the MspI site located in the IGR-1.

Figure 5. Restriction of QY PCR amplified products of wheat stem rust C36(48) with EcoRI and MspI. The 1.3% agarose gel was run at 35 V for 20 h (700 Vh). A) Agarose gel of the restrictions of the QY PCR amplified products with EcoRI, MspI, and EcoRI plus MspI: lane 1, unrestricted; lane 2, EcoRI; lane 3, MspI; lane 4, EcoRI plus MspI; lane 5, BRL 1 kb ladder. B) Southern blot of A) probed with pMF2.

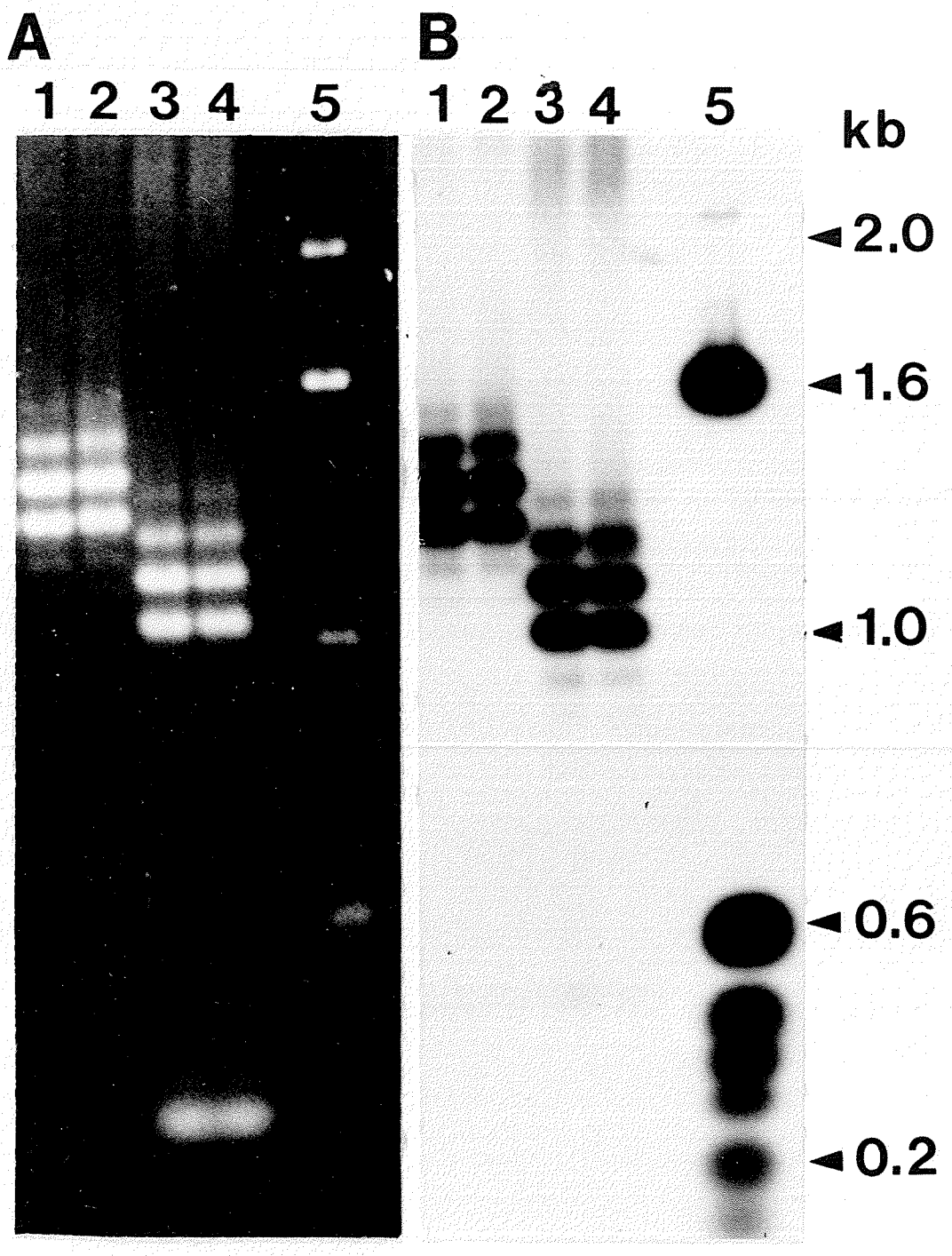
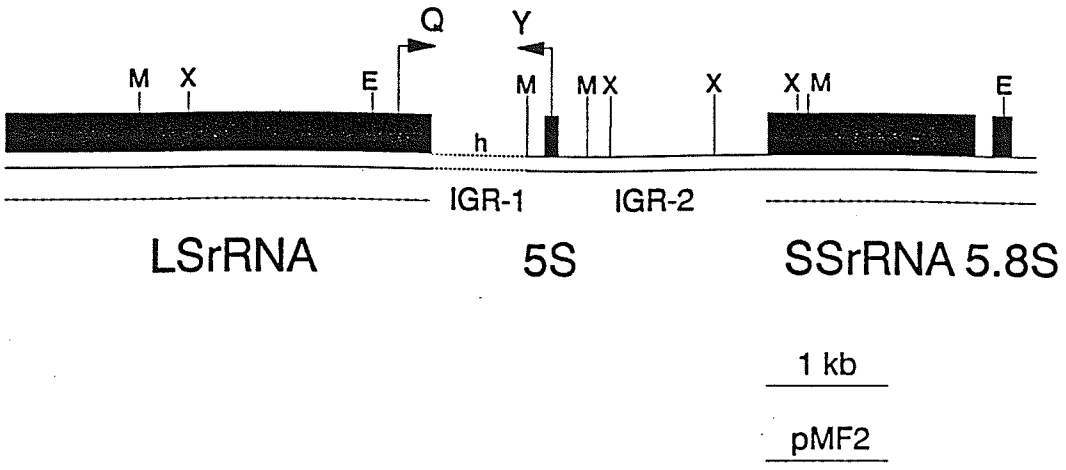

A
1 2 3 4 5
B
1 2 3 4 5
kb
2.0
1.6
1.0
0.6
0.2

Figure 6. Physical map of the rDNA repeat unit in P. graminis f.sp. tritici race C36(48). Raised blocks represent the ribosomal genes (LSrRNA, large subunit rRNA gene; SSrRNA, small subunit rRNA gene; 5.8S, 5.8S rRNA gene, 5S, 5S rRNA gene). IGR-1, DNA fragment between the 3' end of the LSrRNA and the 5' end of the 5S rRNA gene; IGR-2, DNA fragment between the 3' end of the 5S rRNA gene and the 5' end of the SSrRNA. Restriction enzyme sites: E, EcoRI; X, XbaI; and M, MspI. The primer annealing sites and direction of transcription (as indicated by arrow) are shown for the primers Y and Q. The region in which length heterogeneities is indicated by h. The regions which are homologous to the pMF2 probe are indicated by a dashed line. The MspI and EcoRI sites are published results from Kim et al. (1992), the XbaI sites are unpublished results from W. K. Kim.


Q
Y
M
X
E
M
M
X
X
X
M
E
h
IGR-1
IGR-2
LSrRNA
5S
SSrRNA 5.8S
1 kb
pMF2

In general, the 3' end of the LSrRNA genes is highly variable among fungal species. Because of the possibility of this variability at the Q end of the QY PCR product, the starting point of the heterogeneity observed in the QY region of the wheat stem rust races (C36(48) in particular) can not be precisely located. Since the two C36(48) QY fragments and single C1(17) QY fragment were found to be identical in sequence through the terminus of the gene (determined by alignment with the LSrRNA gene of yeast as seen in Fig 7), it may be concluded that heterogeneity is not in the 26S rRNA gene. This places heterogeneity in the IGR-1 downstream of the 3' end of the 26S rRNA gene.

To confirm that the results obtained using PCR amplified DNA accurately reflected native DNA, native DNA of C36(48) was restricted using MspI and MspI plus EcoRI (as was the QY PCR product of C36(48)) and probed with a ^{32}P labelled QY C36(48) PCR product. Examination of the MspI and EcoRI banding patterns seen in Fig 8 shows them to be identical to the banding pattern seen in Fig 5, which features restictions of the heterogeneous QY PCR product of C36(48), the banding patterns differ only in sizes.

To discover if differences exist between isolates of specific races (as currently defined), the QY PCR products of two isolates of C17(56) and five isolates of C53

Figure 7. DNA sequence of the 3' end of the LSrRNA (26S rRNA) genes of P. graminis races C36(48) and C1(17) which are identical. This sequence is aligned with that of the LSrRNA gene in yeast (Gutell and Fox 1988) to determine the 3' end of the 26S rRNA gene.

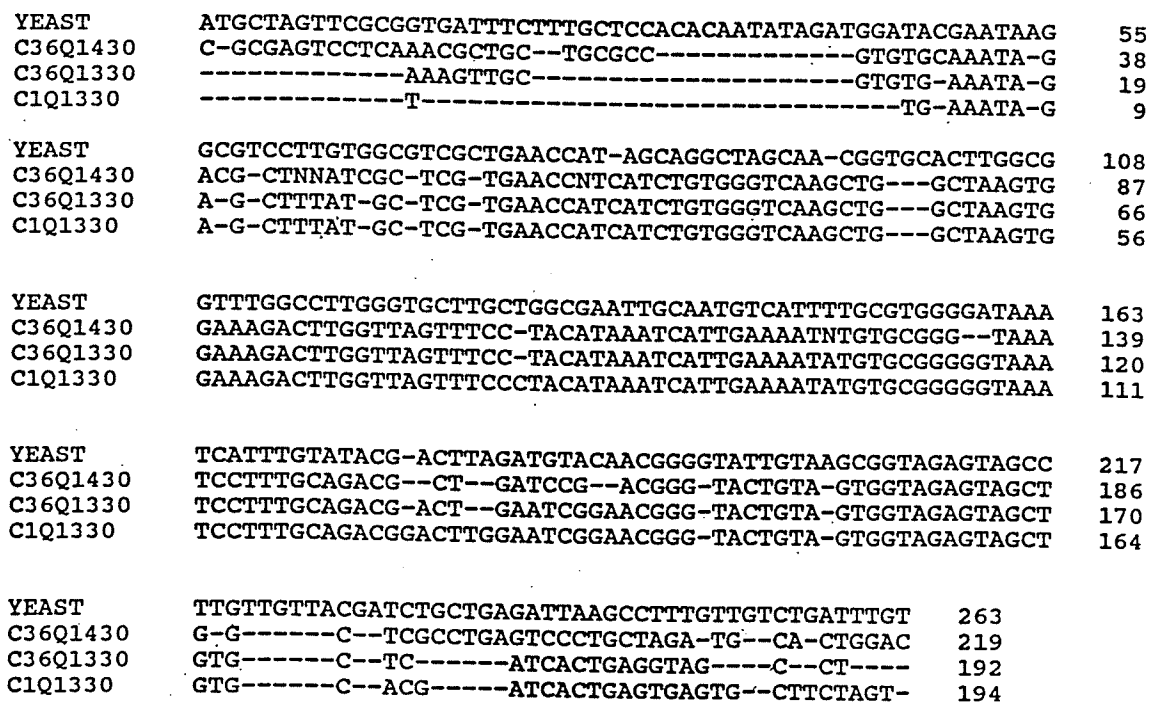
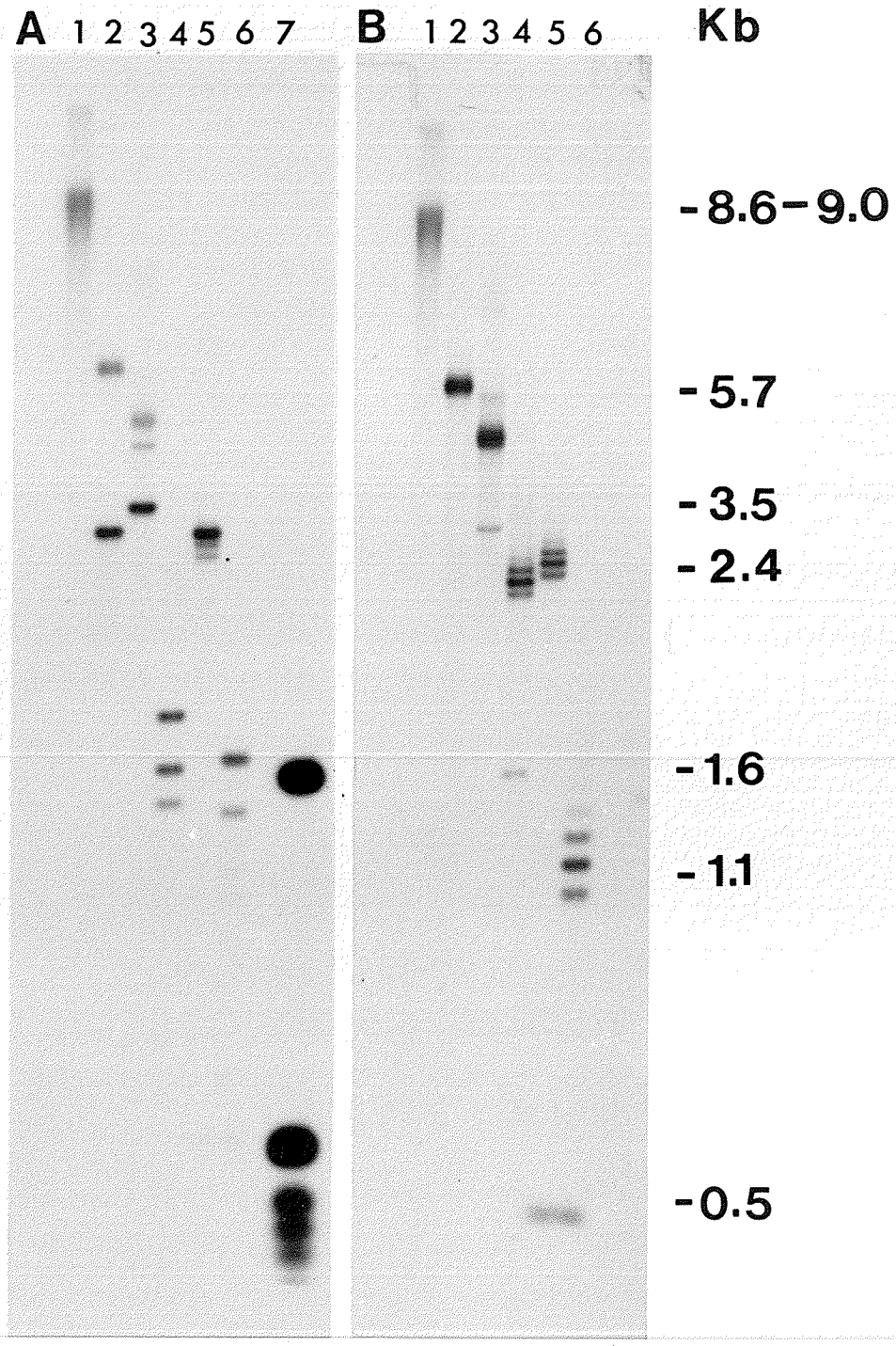

YEAST ATGCTAGTTCGCGGTGATTTCTTTGCTCCACACAATATAGATGGATACGAATAAG 55
C36Q1430 C-GCGAGTCCTCAAACGCTGC--TGCGCC----------GTGTGCAAATA-G 38
C36Q1330 ----------AAAGTTGC----------GTGTG-AAATA-G 19
C1Q1330 ----------T----------TG-AAATA-G 9
YEAST GCGTCCTTGTGGCGTCGCTGAACCAT-AGCAGGCTAGCAA-CGGTGCACTTGGCG 108
C36Q1430 ACG-CTNNATCGC-TCG-TGAACCNTCATCTGTGGGTCAAGCTG---GCTAAGTG 87
C36Q1330 A-G-CTTTAT-GC-TCG-TGAACCATCATCTGTGGGTCAAGCTG---GCTAAGTG 66
C1Q1330 A-G-CTTTAT-GC-TCG-TGAACCATCATCTGTGGGTCAAGCTG---GCTAAGTG 56
YEAST GTTTGGCCTTGGGTGCTTGCTGGCGAATTGCAATGTCATTTTGCGTGGGGATAAA 163
C36Q1430 GAAAGACTTGGTTAGTTTCC-TACATAAATCATTGAAAATNTGTGCGGG--TAAA 139
C36Q1330 GAAAGACTTGGTTAGTTTCC-TACATAAATCATTGAAAATATGTGCGGGGGTAAA 120
C1Q1330 GAAAGACTTGGTTAGTTTCCCTACATAAATCATTGAAAATATGTGCGGGGGTAAA 111
YEAST TCATTTGTATACG-ACTTAGATGTACAACGGGGTATTGTAAGCGGTAGAGTAGCC 217
C36Q1430 TCCTTTGCAGACG--CT--GATCCG--ACGGG-TACTGTA-GTGGTAGAGTAGCT 186
C36Q1330 TCCTTTGCAGACG-ACT--GAATCGGAACGGG-TACTGTA-GTGGTAGAGTAGCT 170
C1Q1330 TCCTTTGCAGACGGACTTGGAATCGGAACGGG-TACTGTA-GTGGTAGAGTAGCT 164
YEAST TTGTTGTTACGATCTGCTGAGATTAAGCCTTTGTTGTCTGATTTGT 263
C36Q1430 G-G------C--TCGCCTGAGTCCCTGCTAGA-TG--CA-CTGGAC 219
C36Q1330 GTG------C--TC------ATCACTGAGGTAG----C--CT---- 192
C1Q1330 GTG------C--ACG-----ATCACTGAGTGAGTG--CTTCTAGT- 194

Figure 8. Southern blot of restricted native DNA of wheat stem rust race C36(48): lanes: 1, unrestricted native DNA; 2, EcoRI; 3, XbaI; 4, EcoRI plus XbaI; 5, MspI; 6, MspI plus EcoRI; 7, BRL 1 Kb ladder. A) Southern blot probed with pMF2, B) Southern blot probed with QY PCR product.


A 1 2 3 4 5 6 7
B 1 2 3 4 5 6
Kb
-8.6-9.0
-5.7
-3.5
-2.4
-1.6
-1.1
-0.5

collected from four different Canadian provinces over a period of 18 years were compared (Fig 9). The isolates were found to be identical within each race.

DNA sequence of the 5'end of the 5S rRNA gene and flanking regions in the cereal rusts

The 3' end of the IGR-1 of two races of wheat leaf rust, two races of wheat stem rust, and one race of oat crown rust, was sequenced using QY PCR product as template with sequencing proceding from Y primer inside the 5S rRNA gene. The QY PCR product of the individuals was run on an agarose gel and the DNA was then eluted from the agarose. Two different sizes of the heterogeneous QY PCR product of C36(48) were used (the 1.38 and 1.48 kb fragments). The sequences are shown in Fig 10. Attempts at sequencing further into the IGR were unsuccessful. Characteristically spacers have an extensive secondary structure which often makes sequencing difficult if not impossible, which might explain the difficulties experienced in sequencing regions of the IGR-1. The DNA sequence of the IGR-1 was found to be virtually identical within the species for the region of the IGR-1 which was sequenced. The largest difference seen between two races of the same species was in wheat stem rust where the spacer regions sequenced were found to be 94% homologous. The two sizes of the QY PCR product of C36(48)

Figure 9. Agarose gel of QY PCR amlified products of various isolates of races C17(56) and C53. Lanes: 1, BRL 1 Kb ladder; 2 and 3, race C17(56) culture no. 233 (collected 1980, Morden, MB); 4, race C17(56) culture no. 273 (collected 1982, Vineland BC; 5, race C53, culture no. 295 (collected 1974, Qu'appelle, SK); 6, race C53, culture no. 29 (collected 1983, Dundas, ON); 7, race C53, culture no. 633 (collected 1978, New Liskeard, ON); 8, race C53, culture no. 2 (collected 1984, Portage la Prairie, MB); 9, race C53, culture no. 96 (collected 1973, Sanford, MB).

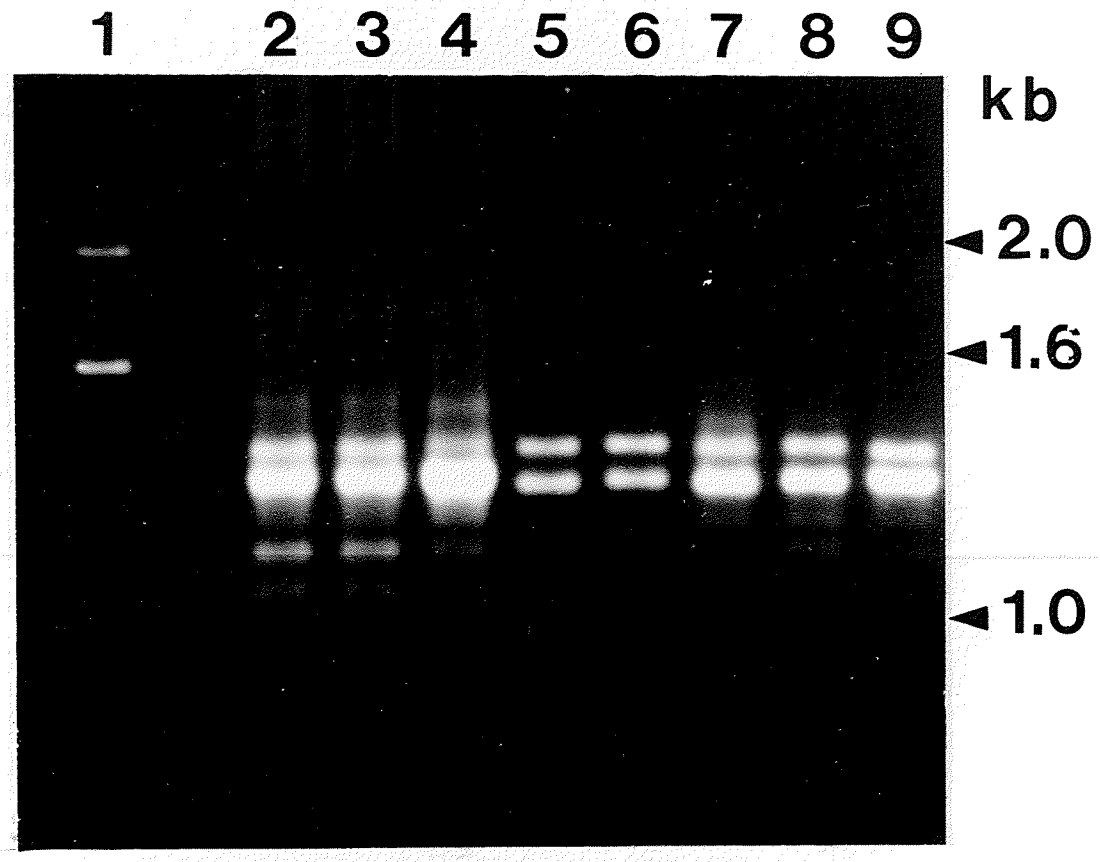

1
2
3
4
5
6
7
8
9
kb
2.0
1.6
1.0

Figure 10. DNA sequences of the 5' end of the 5S rRNA genes and flanking regions (3' end of IGR-1) of a variety of rust races. Sequencing proceeded from Y primer using the QY PCR amplified products of: wheat stem rust races C1(17) and C36(48); wheat leaf rust races Lr1 and Lr9; oat crown rust race 169. The sequence of C36(48) represents the identical sequences of the 1.38 and 1.48 kb QY amplified PCR products. The upstream TATA sequences are underlined. The sequence of the 5' end of the 5S rRNA gene is indicated by bold face print.

```
C1(17)      ----------------------------------------------A----     1
C36(48)     GGGG-----CTTACTCT-----------------GGCTTTTGTCTA----    24
Lr1         AATATGTCAC----CCTATAATAATG---------GCA----ACTAATGG    33
LR9         AATATGTCAC----CCTATAATAATG---------GCA----ACTAATGG    33
OCR 169     GA-ATATCACTTGCCCAATGATGATGTTGGATAGGGCAGGTGACAAAATT    49

C1(17)      ----------CTT-------T--------------C-TGTATGAAAGTAT    19
C36(48)     ----------CTTATCAACTT--------------C-TTTATGAAAGTAT    49
Lr1         GG----CTCAC---TCTGCTTGTTTGTTTGTTAAT---------------    61
Lr9         GG----CTTAC---TCTGCTTGTTTGTTTGTTAAT---------------    61
OCR 169     ATTTTCCATTCATATATACATGTATATATTTTTTTCATATAAGAAAGTAA    99

C1(17)      A-------------------------------------CTTACTCTA-    29
C36(48)     A-------------------------------------CTTACTCTA-    59
Lr1         ------------------------GTTAATCAATGTG-------------    74
Lr9         ------------------------GTTAATCAATGTG-------------    74
OCR 169     TATGGTCACCCTATAACATCTATAGGGAAACAAAATGGGGCTTACTCTGG   149

C1(17)      -----------------AAATCCACC------TCTCACT----GTCATCT    52
C36(48)     -----------------AAATCCACC------TCTCACT----GTCATCT    82
Lr1         CATGTAAGGGTAGGTATATAT--ATCCATAAAACACCCTATATGTTATCT   122
Lr9         CATGTAAGGGTAGGTATATAT--ATCCATAAAACACCCTATATGTTATCT   122
OCR 169     CTTGTGAATGGGGGCTTTATTCGTGCAAAAATTCAAACATCATTTCATCT   199

C1(17)      GGGGCCATACCACAGT    68
C36(48)     GGGGCCATACCACAGT    98
Lr1         GGGGCCATACCACA--   136
Lr9         GGGGCCATACCACA--   136
OCR 169     GGGGCCATACCACA--   213
```

were found to be of identical sequence. The different species of the rusts were found to have essentially different sequences for the 3' end of the IGR-1 making this region an excellent area to study the possibilities of creating species specific diagnostic oligonucleotide probes for rapid identification of the rust species. The sequence of the IGR-1 also features TATA sequences upstream of the 5S rRNA gene (shown in Fig 10) which have been shown previously in the bunts and are now seen in the rusts as well, suggesting regions of possible regulatory significance. The homology of the sequence of the 5S rRNA gene between species is not surprizing as this gene is typical of highly conserved 5S rRNA genes among the fungi. Indeed, the sequence differences among the three cereal rusts further supports findings in an earlier report on genotype diversity among the three cereal rusts by 2-dimensional polypeptide mapping analysis (Kim et al. 1985).

General conclusions

In general, it seems that heterogeneity, at least in the IGR-1, is much more strongly pronounced in wheat stem rust as compared to the other rusts and the bunts examined in this study. A possible explanation for this is that the wheat stem rust races are not as highly evolved as the other

still actively evolving and this is expressed in the form of heterogeneity. The region of heterogeneity has been narrowed to the region of the IGR-1 bounded by the 26S rRNA gene downstream and the MspI site (in the IGR-1) upstream. The various QY PCR products of the individual wheat stem rust races (C36(48) in particular), results of heterogeneity, differ in sizes of approximately 100 bp suggesting that heterogeneity is the result of varying numbers of a 100 bp repeat unit in the IGR-1. There is still work to be done in the IGR-1 to try to pinpoint the region of heterogeneity and to determine if it truly is due to a repeat unit in the IGR-1. Perhaps this region will contain portions which are race specific allowing the production of rapid diagnostic probes to determine quickly the race of the species. This possibility looks to be particularly promising for the wheat stem rust races which are already indicating the value of using PCR products as molecular markers for race identification.

For the first time, the positions of the 5S rRNA genes in both the rusts and bunts have been shown to be in the IGR dividing this region into the IGR-1 and IGR-2. The 5S rRNA gene has been found to be located on the same DNA strand and transcribed in the same direction as the other genes constituting the ribosomal DNA repeat unit in both the rusts and bunts. In sequencing the gene and its flanking regions,

possible sites for regulation as well as sites important for transcription were found in the form of upstream TATA sequences and downstream T-rich sequences. These may provide areas for further study. The area of the IGR-1 just upstream of the 5S rRNA gene was found to vary between species of the rusts as well as between the Puccinia and Tilletia genera suggesting this region is an excellent one to use as template for the production of diagnostic oligonucleotide probes for the rapid identification of species of the rusts. Considering the lack of molecular information on the genome structure of cereal rusts and related species, the data presented in this study could prove to be useful for the study of evolutionary and phylogenetic relationships among species of Basidiomycetes.

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